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Enterotoxigenic Profiles of Food-Poisoning and Food-Borne *Bacillus cereus* Strains

Marie-Hélène Guinebretière,* Véronique Broussolle, and Christophe Nguyen-The

Institut National de la Recherche Agronomique, UMR A408 Sécurité et Qualité des Produits d'Origine Végétale, INRA, Domaine Saint-Paul, Site Agroparc, F-84914 Avignon Cedex 9, France

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The enterotoxigenic profiles of 51 *B. cereus* food-related strains were compared to those of 37 *B. cereus* food-poisoning strains. *cytK* and association of *hbl-nhe-cytK* enterotoxin genes were more frequent among diarrheal strains (73 and 63%) than among food-borne strains (37 and 33%). Unlike diarrheal strains, food-borne strains showed frequent *nhe* and *hbl* gene polymorphisms and were often low toxin producers.

Bacillus cereus can cause food-borne diarrhea by producing heat-labile enterotoxins during growth of vegetative cells in the small intestine (6). Four different enterotoxins have been characterized: two protein complexes, hemolysin BL (HBL) and nonhemolytic enterotoxin (NHE), and two enterotoxic proteins, enterotoxin T (bc-D-ENT) (1) and cytotoxin K (11). HBL complex is composed of three proteins, B, L_1, and L_2 (4, 5) transcribed from the genes *hblC* (encoding L_2), *hblD* (encoding L_1), and *hblA* (encoding B), organized in one operon together with a fourth gene, *hblB* (encoding the B′ protein) (9, 13). NHE complex is also composed of three different proteins, NheA, NheB, and NheC encoded by the three genes *nheA, nheB*, and *nheC*, and it is also organized in one operon (7).

B. cereus is widespread in foods, whereas diarrheal poisoning caused by *B. cereus* is fairly infrequent. The ability to cause diarrhea thus presumably varies among strains. Only few data are available concerning the genetic and toxigenic potential of *B. cereus* strains from vegetable products, although vegetables are frequently contaminated by *B. cereus*. The objective of the present work was to analyze the distribution of enterotoxin genes and enterotoxin production in food strains of various origins (isolated from cooked chilled foods and vegetables) and to compare enterotoxigenic profiles to those in diarrheal food-poisoning strains.

Eighty-eight bacterial strains (51 food-related strains and 37 food-poisoning strains, listed below in Tables 2 and 3) were tested for enterotoxin production, using the *B. cereus* Enterotoxin Reverse Passive Latex Agglutination test kit (Oxoid, Basingstoke, England) and the *Bacillus* Diarrheal Enterotoxin visual immunoassay (Tecra Diagnostics, Roseville, Australia), according to the manufacturers' instructions. Amounts of produced enterotoxin were evaluated with index values derived from the Oxoid and Tecra reading scale (Table 2). All strains were also tested for the presence of the genes *hbl* (C, D, A, and B), *nhe* (A, B, and C), *bceT* (encoding the bc-D-ENT enterotoxin), and *cytK* (encoding cytotoxin K). DNA was extracted as previously described (2). The primers used for the detection of the various genes are presented in Table 1. Amplified products from strains ATCC 14579T, 1230/88, and 391/98 were sequenced to check the specificity of the designed primers. Except for amplifications with *hblB* primers, the PCR mixture (50 μl) consisted of 100 ng of DNA template, 200 μM deoxynucleoside triphosphate mix (Eurogentec S. A., Seraing, Belgium), 2.5 mM $MgCl_2$, a 500 nM concentration of each primer, 0.75 U of AmpliTaq polymerase (Perkin-Elmer, Courtaboeuf, France), and AmpliTaq buffer (Eurogentec). Thermal cycling was carried out in a PCR 9700 thermocycler (Perkin-Elmer) with the following run: a starting cycle of 2 min at 94°C, followed by 35 cycles of 1 min at 94°C, 1 min at the annealing temperature (Table 1), and 2 min at 72°C, and a final extension of 5 min at 72°C. For the *hblB* amplification reaction (50 μl), 500 μM deoxynucleoside triphosphate mix, 5 mM $MgCl_2$, and a 300 nM concentration of each primer were used instead of the concentrations stated above. Parameters determined with the PCR 9700 thermocycler were 2 min at 94°C followed by 10 cycles of 10 s at 94°C, 30 s at 58°C, and 2 min at 68°C; 20 cycles of 10 s at 94°C, 30 s at 58°C, 2 min (plus 20 s per cycle) at 68°C; and a final extension at 68°C for 7 min. Strains that were negative in PCR experiments were submitted to Southern blotting to check for the absence of *hbl*, *nhe*, and *cytK*. Positive controls were included for each tested probe. Five micrograms of genomic DNA was digested to completion with *Eco*RI and electrophoresed overnight on a 1% agarose gel at 15 V. Southern blotting was carried out according to standard protocols (14). Probes were generated by PCR amplifications of *hblC*, *hblD*, *hblA*, *nheA*, *nheB*, *nheC*, and *cytK* gene fragments, and ^{32}P-labeling was performed with Ready-to-Go DNA labeling beads (Amersham Pharmacia Biotech, Orsay, France) according to the manufacturer's recommendations. Membranes were hybridized at 60°C for 2 h in Rapid hybridization buffer (Amersham Pharmacia Biotech), washed according to supplied protocols, and exposed to phosphorimager screens (Storm; Molecular Dynamics, Bondoufle,

* Corresponding author. Mailing address: INRA, UMR A408 INRA-Université d'Avignon, Domaine St-Paul, Site Agroparc, 84914 Avignon Cedex 9, France. Phone: (33) 4 32 72 25 24. Fax: (33) 4 32 72 24 92. E-mail: guinebre@avignon.inra.fr.

TABLE 1. Characteristics of primers used in this study[a]

Primer[b]	Gene	Annealing temp for the primer couple (°C)	Amplified fragment size (bp)	Sequence (5′ → 3′)	Primer position	Sequence from which the primer was deduced	
						Origin of DNA sequence	EMBL/GenBank accession no.
HC F	*hblC*	58	740	GATAC(T, C)AATGTGGCAACTGC	40–59	*B. cereus* ATCC 14579[T]	AJ007794
HC R				TTGAGACTGCTCG(T, C)TAGTTG	761–780		
HD F	*hblD*	58	829	ACCGGTAACACTATTCATGC	970–989		
HD R				GAGTCCATATGCTTAGATGC	1780–1799		
HA F	*hblA*	56	1,154	AAGCAATGGAATACAATGGG	1951–1970		
HA R				AGAATCTAAATCATGCCACTGC	3084–3105		
HA F	*hblB*	58	2,684	AAGCAATGGAATACAATGGG	1951–1970		
HB R				AATATGTCCCAGTACACCCG	4616–4635		
NA F	*nheA*	56	755	GTTAGGATCACAATCACCGC	430–449	*B. cereus* 1230/88	Y19005
NA R				ACGAATGTAATTTGAGTCGC	1166–1185		
NB F	*nheB*	54	743	TTTAGTAGTGGATCTGTACGC	1682–1702		
NB R				TTAATGTTCGTTAATCCTGC	2406–2425		
NC F	*nheC*	54	683	TGGATTCCAAGATGTAACG	2935–2953		
NC R				ATTACGACTTCTGCTTGTGC	3599–3618		
BCET1 F	*bceT*	55	661	CGTATCGGTCGTTCACTCGG	1191–1210	*B. cereus* B-4ac	D17312
BCET3 R				GTTGATTTTCCGTAGCCTGGG	1832–1852		
BCET1 F	*bceT*	55	924	CGTATCGGTCGTTCACTCGG	1191–1210		
BCET4 R				TTTCTTTCCCGCTTGCCTTT	2096–2115		
CK F	*cytK*	54	809	ACAGATATCGG(G, T)CAAAATGC	1859–1878	*B. cereus* 391/98	AJ277962
CK R				GAACTG(G, C)(A, T)AACTGGGTTGGA	2649–2668	*B. thuringiensis* 407[c]	

[a] Primers used to amplify the *bceT* gene have been described by Hansen and Hendriksen (8).
[b] F, forward primer; R, reverse primer.
[c] Sequence from D. Lereclus, Institut Pasteur, Paris, France.

France) before revelation. The Kolmogorov-Smirnov two-sample test was used to determine whether the frequency and association of genes and the production of enterotoxins were significantly different between food-poisoning strains and food-borne strain groups. The test was performed using SYSTAT (version 9.0; SPSS Inc., Chicago, Ill.).

Detection of enterotoxin genes. For the 88 *B. cereus* strains, *hbl* and *nhe* gene detection by both PCR and Southern blotting methods correlated well with the results of the Oxoid and Tecra tests, respectively (Tables 2 and 3). Among the 37 food-poisoning strains, none needed confirmation by Southern analysis for *hbl* genes (C, D, and A), which were easily detected by PCR, and only eight strains (22%) negative in PCR for one or two of the *nhe* genes were found to be positive by Southern blotting (Table 2). This contrasted with results obtained for the 47 and 49 food-borne strains that were positive for the HBL and NHE enterotoxins, respectively: 17 strains (36%) and 31 strains (63%) negative in PCR for one or two of the respective *hbl* and *nhe* genes were found to be positive by Southern blotting (Table 3). This indicates a high polymorphism in the *hbl* and *nhe* sequences among food-associated strains, which differed markedly from food-poisoning strains in that respect. Southern analysis showed that genetic heterogeneity among *B. cereus* strains, and particularly among environmental strains, is more truly associated with sequence polymorphism than with the lack of one of the genes composing the *hbl* or *nhe* operon.

Distribution of enterotoxin genes. Almost all the strains from the food-poisoning and food-borne ecosystems carried *nhe* genes, as observed in previous works (8, 12). The *bceT* gene was widely distributed (57 and 71% among food-poisoning and food-borne strains, respectively). The *hbl* (C, D, and A) genes were also highly frequent in the two groups of strains (73 to 92%). The *cytK* gene was frequently detected among diarrheal strains, 73% of which carried it. It was present at a significantly ($P = 0.007$) lower frequency among food-borne strains; only 37% of the food strains were concerned. Stenfors and Granum (15) found three strains (13%) carrying this gene among 23 strains from dairy products. Profiles associating the *nhe*, *hbl*, and *cytK* genes were significantly ($P = 0.049$) more frequent among food-poisoning strains (63% of the strains concerned) than among food-borne strains (33% of the strains).

Enterotoxin levels produced among strain samples. Among strains carrying *hbl* genes, the percentage of those producing high levels of enterotoxin HBL (with an index of ≥128) was significantly ($P = 0.002$) higher for food-poisoning strains (74%) than for food-borne strains (32%). Similarly, among strains carrying *nhe* genes, the frequency of high enterotoxin NHE producers (production at an index of ≥4) was 78% for food-poisoning strains and only 20% for food-related strains. Furthermore, 11 strains (22%) among the 49 food-borne strains carrying *nhe* genes did not produce or only weakly produced enterotoxin NHE (index < 3). This emphasizes a difference in gene expression between food-borne and food-poisoning strains. Psychrotrophic strains isolated from pasteurized milk by in't Veld et al. (10) were also low producers of enterotoxin BL, with only 26 strains out of 86 (30%) exhibiting an index of ≥128 in the Oxoid test. Beattie and Williams (3) also observed wide variations in the amount of enterotoxin produced from dairy-associated strains.

TABLE 2. Characteristics of food-poisoning strains

Strain	HBL complex[a]				Oxoid kit test index[b]	NHE complex[a]			Tecra kit test index[c]	*cytK*[a,d]	*bceT*[a]
	hblC	*hblD*	*hblA*	*hblB*		*nheA*	*nheB*	*nheC*			
0861/00	−	−	−	−	0	+	+	+[s]	4	−	−
DSM 2301	−	−	−	−	0	+[s]	+[s]	+	3.5	−	+
LMG 17604	−	−	−	−	0	+[s]	+	+	3	−	+
391/98	−	−	−	−	0	−	−	−	2	+	+
0500/00	−	−	−	−	0	+	+	+	4.5	−	−
F2404B/79	−	−	−	−	0	+	+	+[s]	4	−	+
F0285/78	−	−	−	−	0	+	+	+	3	+	−
F2085/98	−	−	−	−	0	+[s]	+	+	4.5	+	−
DSM 8438	−	−	−	−	0	+	+	+	4	+	−
F4346/75	−	−	−	−	0	+	+	+	4	−	−
F528/94	+	+	+	−	16	+	+	+	4	−	+
IH41064	+	+	+	+	128	+	+	+	4	+	−
F3465/73	+	+	+	+	64	+	+	+	3.5	+	−
F3371/93	+	+	+	+	>128	+	+	+	5	+	−
F0210/76	+	+	+	+	128	+	+	+	4	+	+
F4096/73	+	+	+	+	64	+	+	+	3.5	+	+
F4094/73	+	+	+	+	64	+[s]	+	+	3	+	+
F3453/94	+	+	+	+	32	+	+	+	4	+	+
F4370/75	+	+	+	+	>128	+	+	+	4	−	−
DSM 4222	+	+	+	−	>128	+	+	+	4	−	−
F2769/77	+	+	+	−	>128	+	+	+	4.5	−	+
F4433/73	+	+	+	+	>128	+	+	+	5	+	−
1230/98	+	+	+	+	128	+	+	+	4	+	−
F4815/94	+	+	+	+	32	+	+	+	3	+	−
LMG 17605	+	+	+	+	16	+	+	+[s]	4.5	+	−
LMG 17615	+	+	+	+	128	+	+[s]	+[s]	5	+	−
F4430/73	+	+	+	+	>128	+	+	+	4.5	+	+
F1942/85	+	+	+	+	128	+	+	+	4	+	+
F4432/73	+	+	+	+	>128	+	+	+	4	+	+
DSM 4282	+	+	+	+	128	+	+	+	3.5	+	+
0230/00	+	+	+	+	>128	+	+	+	4	+	+
1651/00	+	+	+	+	128	+	+	+	4	+	+
98HMPL63	+	+	+	+	>128	+	+	+	4	+	+
F284/78	+	+	+	+	>128	+	+	+	4	+	+
F352/90	+	+	+	−	>128	+	+	+	4	+	+
F2081A/98	+	+	+	−	>128	+	+	+	4	+	+
F2081B/98	+	+	+	+	>128	+	+	+	4	+	+

[a] +, a PCR product of the expected size was observed; −, no PCR product was observed; +[s], no PCR product was observed but the gene was detected by Southern blotting.

[b] For the Oxoid test, the indices from 0 to 128 corresponded to the last supernatant dilution rate (among 1/2 serial dilutions) for which enterotoxin remained detectable. According to the manufacturer's instructions, strains with an index of 0 were considered negative.

[c] For the Tecra test, indices from 1 to 5 corresponded to the coloration intensity. According to the manufacturer's instructions, strains with an index of <3 were considered negative.

[d] *cytK* not detected by PCR was always confirmed to be absent by Southern blotting.

TABLE 3. Characteristics of food-borne strains

Strain	HBL complex[a]				Oxoid kit test index[b]	NHE complex[a]			Tecra kit test index[c]	*cytK*[a,d]	*bceT*[a]
	hblC	*hblD*	*hblA*	*hblB*		*nheA*	*nheB*	*nheC*			
PA	−	−	−	−	0	+	+	+	2.5	+	+
C74	−	−	−	−	0	+[s]	+	+	3.5	−	−
C39	−	−	−	−	0	+[s]	+	+	4.5	−	−
C33	−	−	−	−	0	+	+	+[s]	3.5	+	+
SL′	+[s]	+	+	−	16	+[s]	+	+	3	−	−
SV	+[s]	+	+	−	32	+[s]	+	+	2.5	−	−
5	+[s]	+[s]	+[s]	−	4	+[s]	+	+	2	−	−
BC	+	+	+	+	16	+	+	+[s]	3	−	−
PF	+	+	+	+	64	+[s]	+	+	3.5	−	−
C1	+[s]	+[s]	+[s]	−	2	+	+	+[s]	3	−	−
C35	+[s]	+[s]	+	−	64	+	+	+	2.5	−	−
C36	+[s]	+[s]	+	+	64	−	−	−	2	−	−
C46	+[s]	+[s]	+[s]	−	1	+[s]	+	+[s]	3	−	−
C64	+[s]	+	+	−	16	+	+[s]	+[s]	2.5	−	−
I3	+[s]	+	+	−	1	−	−	−	2	−	−
1	+	+	+	+	2	+	+	+[s]	3	−	+
11	+	+	+	+	16	+[s]	+	+	3.5	−	+
15	+	+	+	+	128	+	+	+	4.5	−	+
25	+	+	+	+	128	+	+	+	4	−	+
BK	+	+	+	−	4	+[s]	+	+	2	−	+
BL	+[s]	+	+	−	8	+	+	+	3	−	+
BN	+	+[s]	+[s]	−	0	+	+	+	3.5	−	+
BX	+	+	+	−	4	+	+	+[s]	3	−	+
BS	+	+	+	+	8	+	+	+	2.5	−	+
SO	+	+	+	+	4	+	+	+[s]	3	−	+
SZ	+	+	+	+	4	+[s]	+	+[s]	3	−	+
SB′	+	+	+	+	>128	+[s]	+	+	4	−	+
SD	+	+	+	−	16	+[s]	+	+	3.5	−	+
SM	+	+	+	+	64	+[s]	+	+[s]	2.5	−	+
C41	+[s]	+	+	−	2	+[s]	+	+	1.5	−	+
I6	+[s]	+[s]	+[s]	−	2	+[s]	+	+[s]	4	−	+
I11	+	+	+	−	128	+	+	+	3.5	−	+
I20	+[s]	+	+	−	16	+	+[s]	+	3	−	+
I21	+[s]	+[s]	+	−	16	+[s]	+	+	3	−	+
C43	+	+	+	−	128	+	+	+[s]	5	+	−
I22	+	+	+	−	128	+	+	+	4	+	−
32	+	+	+	+	>128	+[s]	+	+	4	+	+
BC′	+	+	+	+	64	+	+[s]	+	3	+	+
A3	+	+	+	+	16	+[s]	+	+	3.5	+	+
C3	+	+	+	+	128	+	+	+	3.5	+	+
C15	+	+	+	+	128	+	+	+	3.5	+	+
C38	+	+	+	−	>128	+	+	+	4	+	+
C57	+	+	+	−	32	+	+	+	3.5	+	+
I2	+	+	+	−	64	+	+	+	3.5	+	+
I7	+[s]	+	+	−	64	+	+	+	3.5	+	+
I10	+	+	+	−	128	+	+	+	3.5	+	+
I12	+	+	+	−	>128	+[s]	+	+	3.5	+	+
I13	+[s]	+	+	−	128	+	+	+	5	+	+
I16	+	+	+	−	>128	+	+[s]	+	3.5	+	+
I17	+	+	+	−	128	+	+	+	2.5	+	+
I23	+	+	+	+	32	+[s]	+	+	2.5	+	+

[a] +, a PCR product of the expected size was observed; −, no PCR product was observed; +[s], no PCR product was observed but the gene was detected by Southern blotting.

[b] For the Oxoid test, the indices from 0 to 128 corresponded to the last supernatant dilution rate (among 1/2 serial dilutions) for which enterotoxin remained detectable. According to the manufacturer's instructions, strains with an index of 0 were considered negative.

[c] For the Tecra test, indices from 1 to 5 corresponded to the coloration intensity. According to the manufacturer's instructions, strains with an index of <3 were considered negative.

[d] *cytK* gene not detected by PCR was always confirmed to be absent by Southern blotting.

Some significant differences were found in the genetic and toxigenic potential of food-poisoning strains and food-borne strains, supporting the hypothesis that many food-borne strains are less prone to cause diarrhea.

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